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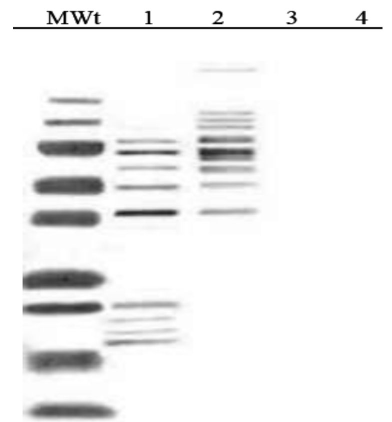
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Product Name
Mouse Anti-Flavobacterium psychrophilum Horseradish Peroxidase Conjugated Monoclonal Antibody

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CAT No.
IPA116H

LOT No.
23590C

Quantity
100µg

F. psychrophilum ELISA Working Protocol

Supplies

-0.5 M sodium carbonate-bicarbonate buffer, pH 9.6 (Dissolve 1.59 g Na2CO3 and 2.59 g NaHCO3 in 900 ml DI H2O, adjust pH to 9.6, and bring volume up to 1 L. This should be made fresh every two weeks.)

- PBS, pH 7.2
- 5% (w/v) non-fat dry milk + PBS, pH 7.2 (NFDM-PBS) (Bio-Rad #170-6404)*
- 0.05% (v/v) Tween-20 + PBS, pH 7.2 (PBST)*
- 1 N HCl
- TMB One Component HRP Microwell Substrate (SurModics #TMBW 0100-01)
- Nunc MaxiSorp 96-well plates (Fisher Scientific #12-565-135)
- MAb FL43 (ImmunoPrecise #)
- MAb FL43 conjugated to HRP (ImmunoPrecise #IPA116H)

*Both PBST and NFDM-PBS should be made fresh the day of the ELISA.

Plate Setup and Coating

1. Set-up the layout of the plate. There is one full control plate for every 4 plates run. On the control plate, there should be 4 controls, at least four replicates of each. There should also be a standard curve. All samples should be run in duplicate (Figure 1).
   a. Blank – These wells receive PBST in lieu of sample.
   b. Substrate Control – Wells receive coating solution only and PBS throughout the entire procedure until the point of TMB addition.
   c. Chromogen Control – Wells receive coating solution only and PBS in lieu of samples.
   d. Negative Control – This is tissue sampled from fish found to be free of *F. psychrophilum*. Currently, this is checked by sampling kidney, liver, and spleen from a random sample of the population and checking for yellow-pigmented bacterial growth.
   e. Standard Curve – An exponentially growing culture of *F. psychrophilum* is diluted from 10-1 to 10-6 in homogenized tissue from negative control fish. An undiluted sample of the bacteria is also run.
2. Every subsequent plate should have the negative control and blank wells included.

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Caution: Contains Sodium Azide

3. The day before running the ELISA, dilute purified MAb FL43 to a working concentration of 3 µg
ml^{-1} in 0.5 M sodium carbonate-bicarbonate coating buffer.
4. Dispense 50 µl of antibody to each well that will have a sample.
5. Wrap the plate in plastic wrap to avoid evaporation and incubate overnight 4°C.

Sample Preparation*

1. Weigh out the desired amount of tissue and add PBST for a final concentration of 1:5 (w/v) for kidney, e.g. 1 gram of kidney and 4 ml of PBST, or 1:8 (w/v) for spleen tissue.
2. Homogenize sample thoroughly.
3. Filter sample through a stainless steel mesh filter.
4. Incubate the filtered homogenate in a waterbath at 55°C for 5 minutes.
5. After incubation, keep the sample on ice until needed for no more than 2 hours.

* Samples should be prepared the morning that the ELISA will be run. Variability increaseswhen samples are homogenized and re-frozen before use.

ELISA

1. The next day, remove the coating antibody from the wells. Gently blot the plate on a paper towel to remove excess fluid.
2. Wash each well with 200 µl of PBST four times. Each PBST wash should last approximately 30 seconds. Between each wash, blot the plate on a paper towel to remove excess liquid.
3. Add 200 µl of NFDM-PBS to each well. Wrap plates in plastic wrap and incubate at room temperature for 30 minutes.
4. Remove the NFDM-PBS from the wells and blot plate on a paper towel.
5. Repeat step 2, washing each well 4 times with PBST for 30 seconds per wash and blotting plate between each wash.
6. Add 50 µl of sample to each well. Blank wells receive 50 µl of PBST, and SC and CC wells receive 50 µl of PBS. These wells should be done first and sealed before adding samples to avoid accidental contamination.
7. Wrap the plate in plastic wrap and incubate at room temperature for 2 hours.
8. After incubation is complete, remove the sample from each well and blot the plate on paper towel.
9. Add 200 µl of NFDM-PBS to each well and let incubate for 30 seconds. Remove the NFDM-PBS from each well and repeat the rinse three more times with a 30 second incubation time for each rinse, blotting the plate between each rinse.
10. Dilute the HRP conjugated antibody to a concentration of 1:250 in PBS.
11. Add 50 µl of diluted HRP conjugated antibody to each well except the SC wells. To those wells, add 50 µl of PBS.
12. Wrap plate in plastic wrap and incubate in the dark at room temperature for 1 hour.

13. After 1 hour, remove the HRP antibody from each well and add 200 µl of PBST to each well. Incubate for 30 seconds before removing the PBST. Repeat this rinse 3 more times. Plates should be gently blotted after each rinse.
14. Add 50 µl of TMB substrate to each well and incubate the plate at room temperature for 8 minutes.
15. To stop the reaction, add 50 µl of 1 N HCl to each well. Read the plate at 450 nm within 1 hour.

Data Analysis

1. To determine the positive-negative threshold, first calculate the average and standard deviation for all negative control wells from all plates. The threshold is the mean of the negative control + 2 standard deviations.
2. The O.D. values for the SC wells should be less than 0.050 and less than 0.060 for the CC wells.
3. Calculate the average and standard deviations for all other samples. The coefficient of variation should also be calculated. The CV should be below 10%, ideally it will be less than 5%.

Figure 1. Plate Layout

First Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	NC		Undil	10^{-4}		1	5	9	13	17	21
B	BLK	NC		Undil	10^{-4}		1	5	9	13	17	21
C	BLK	NC		10^{-1}	10^{-5}		2	6	10	14	18	22
D	BLK	NC		10^{-1}	10^{-5}		2	6	10	14	18	22
E	SC	CC		10^{-2}	10^{-6}		3	7	11	15	19	23
F	SC	CC		10^{-2}	10^{-6}		3	7	11	15	19	23
G	SC	CC		10^{-3}			4	8	12	16	20	24
H	SC	CC		10^{-3}			4	8	12	16	20	24

Subsequent Plates

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK		25	29	33	37	41	45	49	53	57	61
B	BLK		25	29	33	37	41	45	49	53	57	61
C	BLK		26	30	34	38	42	46	50	54	58	62
D	BLK		26	30	34	38	42	46	50	54	58	62
E	NC		27	31	35	39	43	47	51	55	59	63
F	NC		27	31	35	39	43	47	51	55	59	63
G	NC		28	32	36	40	44	48	52	56	60	64
H	NC		28	32	36	40	44	48	52	56	60	64

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